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The impact of repeated organophosphorus pesticide exposure on biomarkers and neurobehavioral outcomes among adolescent pesticide applicators

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Abstract

Egyptian adolescents are hired as seasonal workers to apply pesticides to the cotton crop and may perform this occupation for several years. However, few studies examined the effects of repeated pesticide exposure on health outcomes. The goal of this study was to determine the impact of repeated pesticide exposure on neurobehavioral (NB) performance and biomarkers of exposure (urinary metabolite) and effect (cholinesterase activity). Eighty-four adolescents from two field stations in Menoufia, Egypt, were examined four times: before and during pesticide application season in 2010 and again before and during application season in 2011. At each of the four time points, participants completed a questionnaire, performed an NB test battery, and were assessed for urinary levels of the chlorpyrifos metabolite TCPy (3,5,6-trichloro-2-pyridinol) and blood cholinesterase activity. Following the study cohort over two consecutive pesticide application seasons revealed that TCPy levels significantly increased following exposure, and returned to

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Conflict of interest

Oregon Health and Science University and Dr. Rohlman have a significant financial interest in Northwest Education Training and Assessment, LLC, a company that may have a commercial interest in the results of this research and technology. This potential conflict of interest was reviewed by the University of Iowa and Oregon Health and Science University and an approved conflict of interest in research management plan was implemented.

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baseline levels following the end of the application season. Blood butyryl cholinesterase activity exhibited a similar pattern. Although NB outcomes displayed learning and practice effects over time, deficits in performance were significantly associated with increased TCPy levels with reduction in the number of NB measures showing improvement over time. Biomarkers of exposure and effect demonstrated changes associated with pesticide application and recovery after application ended. Deficits in NB performance were correlated with elevated pesticide exposure. Data demonstrated that repeated pesticide exposure may exert a long-term adverse impact on human health.

Introduction

There is evidence of neurotoxicity of chronic low-to-moderate level exposure to organophosphorus (OP) pesticides among adult farmworkers and pesticide applicators. These studies demonstrated an association between occupational exposure and biomarkers of exposure such as urinary 3,5,6-trichloro-2-pyridinol (TCPy, a chlorpyrifos (CPF) metabolite) (Fenske et al. 2003; Hines and Deddens 2001; Steenland et al. 2000) and effect (cholinesterase activity) (Farahat et al. 2003; Srivastava et al. 2000), neurobehavioral (NB) performance (Bazylewicz-Walczak, Majczakowa, and Szymczak 1999; Farahat et al. 2003; Fiedler et al. 1997; Gomes et al. 1998; Kamel et al. 2003; Mackenzie Ross et al. 2010; Rohlman et al. 2007; Roldan-Tapia et al. 2005; Stephens et al. 1995), and self-reported symptoms (Farahat et al. 2003; London et al. 1998; Ohayo-Mitoko et al. 2000; Smit et al. 2003). However, the majority of investigations relied upon a single time-point comparison between an exposed and an unexposed group. Further, few studies examined the impact of exposure among adolescent (age 14–24, (Geiger and Castellino 2011)) applicators, although those investigations noted similar findings (Abdel Rasoul et al. 2008; Eckerman et al. 2007; Ismail et al. 2017; Rohlman et al. 2014).

As farmworkers and pesticide applicators typically apply pesticides in repeated cycles and across multiple seasons, there is a need to examine the influence of repeated exposure on biomarkers and NB outcomes. However, only a few investigators examined the impact of repeated exposure (Arcury et al. 2009; Baldi et al. 2011; Berent et al. 2014; Garabrant et al. 2009; Quandt et al. 2010). Arcury and colleagues (Arcury et al. 2009) reported changes in the proportion of farmworkers with detectable levels of metabolites across the agricultural season, although these concentrations were not associated with (1) total hours worked, (2) receiving pesticide safety training, (3) number of individuals living in the camp, or (4) number of times hands were washed per days worked. Cholinesterase activity of the farmworkers was also altered from one period to the next; but, the only significant predictor of cholinesterase activity was the number of OP and carbamate pesticide metabolites detected in urine (Quandt et al. 2010). In another study, a higher percentage of the exposed group showed inhibition of cholinesterase activity over the course of a year than controls (Garabrant et al. 2009). Although these studies demonstrated changes in biomarkers over time, investigators did not collect information regarding spray events including times of pesticide application, which limit understanding the relationship between exposure and outcomes.

A few studies examined NB outcomes among exposed groups over time. Unlike biomarkers such as urinary metabolite levels or cholinesterase activity which may display recovery after exposure ends, NB performance may continue to show improvement over time due to learning or practice effects (Baldi et al. 2011; Berent et al. 2014; Butler-Dawson et al. 2016; Nguyen et al. 2015). However, while improvement may be found in both exposed and control groups, often controls demonstrate a greater improvement than exposed subjects (Baldi et al. 2011; Butler-Dawson et al. 2016; Nguyen et al. 2015). None of the previous investigations examined on a repeated basis NB performance of pesticide workers in relation to spray event; thus, there is a need to determine the impact of repeated exposure on both biomarkers of exposure and NB performance among populations exposed to pesticides.

Data presented here are part of a longitudinal study conducted in agricultural districts located in the Menoufia Governorate, Egypt, during 2010 and 2011. This investigation assessed the biomarkers of exposure and effect (Crane et al. 2013), neurological symptoms (Khan et al. 2014), and NB performance (Rohlman et al. 2016) among adolescents. For the purpose of this study, four time points over a 2-year period were selected for comparison, times prior to OP application cycle (early June 2010 and late May 2011) and during OP application cycle (July 2010 and 2011) (Table 1). These time points enabled us to (1) compare NB performance and exposure prior to and during the application seasons across two application periods, (2) examine the influence of pesticide exposure on biomarkers of exposure and effect and NB performance, (3) determine recovery after exposure ends, and (4) impact of repeated exposure. The hypothesis underlying this investigation is that exposure to pesticides might affect the levels of biomarkers, and these amounts might be restored to baseline levels after the end of exposure; on the other hand, NB performance may show an improvement over time, with decrement of NB performance with increasing amounts of exposure (TCPy levels) and fall in number of NB measures exhibiting improvement over time.

Methods

The pesticide application schedule for the cotton crop in Egypt followed a similar schedule for many years, with only minor variations depending upon pest infestation. Pesticide application occurs in four cycles beginning in May and ending in August. Biological growth stimulator is first applied, then an OP pesticide, primarily CPF, is applied, followed by one of the pyrethroid pesticides such as alpha α -cypermethrin or γ -cyhalothrin, and finally another cycle of OP pesticide application (Rohlman et al. 2016). During each cycle, application occurs daily in the afternoon for approximately 5 hr each day. Adolescents are hired during the summer to apply pesticides to the cotton crop under supervision and management of the Ministry of Agriculture. Adolescents apply pesticides primarily using backpack sprayers and may also participate in other tasks such as pesticide mixing, cleaning and maintaining equipment, or holding flags to mark the boundaries of the fields.

Recruitment and data collection

Participants were recruited from two districts (Al-Shohada and Berket El-Sabea') in the Menoufia Governorate, Egypt (Callahan et al. 2014; Crane et al. 2013; Khan et al. 2014;

Rohlman et al. 2016). All pesticide applicators between 12 and 21 years of age from both districts were invited to participate in the study and 100% accepted. Adolescents from the same villages, of similar ages, who did not work for the Ministry of Agriculture were also recruited to participate. However, several of these nonapplicator participants may have applied pesticides outside of the Ministry of Agriculture in family fields, as private applicators, or at home. Exclusion criteria included history of diagnosed neurological or kidney diseases; however, none of the participants reported these conditions. Only participants who completed at least two of the four test sessions were included in this analysis.

Test sessions were conducted at the main field station in each district. During each session, a questionnaire was completed by each participant, containing items addressing demographics, work, family, and health characteristics. A battery of tests were used to assess NB functions, and urine and blood samples were collected for respective measurements of TCPy levels and cholinesterase activity. Each test session consisted of 2 days of testing; the first day was conducted at Al-Shohada, followed by testing at Berket El-Sabea'. Data were collected during the morning prior to the pesticide application in the afternoon (Table 1). All participants or their legal guardians signed informed consent forms. Compensation for all participants of approximately 1 day's salary was provided for each test session completed. Approval of the study was obtained from both the Institutional Review Board at Oregon Health and Science University in June 2009 and by the Medical Ethics committee of the Faculty of Medicine at Menoufia University in July 2009.

NB testing

A range of NB functions were assessed using both computerized and individually administered tests. The Behavioral Assessment and Research System (BARS) was employed to administer the computer-based tests (Rohlman et al. 2003). Tests were selected based upon previous studies with adolescents and adults occupationally exposed to OP pesticides (Abdel Rasoul et al. 2008; Farahat et al. 2003; Kamel et al. 2003; Rohlman et al. 2014; Roldan-Tapia et al. 2005; Stephens et al. 1995). A battery of tests were used to assess a range of NB functions such as attention, response speed, memory, and coordination (Table 3).

TCPy analysis

Urine samples were collected from the study participants at each time point to assess the levels of TCPy, a specific metabolite of CPF. Samples were transported from fields to Menoufia University Labs in Shebin Elkom, Egypt, in a cooler with wet ice. Samples were stored at -20°C until they were shipped to the University at Buffalo (Buffalo, NY, USA) on dry ice for analysis. The method of TCPy analysis was described elsewhere (Farahat et al. 2010). This method utilizes negative-ion chemical ionization gas chromatography-mass spectrometry and $^{13}\text{C}-^{15}\text{N}-3,5,6\text{-TCPy}$ as an internal standard (Farahat et al. 2010). Urine TCPy concentrations are presented as mg TCPy/g creatinine. Jaffe reaction was used to measure creatinine concentrations (Fabiny and Ertingshausen 1971). The precision and

reliability of the method are high with an intraclass correlation coefficient of 0.997, and a coefficient of variation <2%.

Cholinesterase analysis

Blood samples were collected to measure cholinesterase activity at selected time points (Table 1). Ten-milliliters of lavender top (EDTA) vacutainer tubes were used to collect blood samples. The tubes were then placed immediately on wet ice and transported to Menoufia University to be analyzed. An EQM Test-Mate kit (EQM Research, Cincinnati, OH, USA) was employed to analyze acetyl cholinesterase (AChE) and butyryl cholinesterase (BChE) activity twice as described elsewhere (Farahat et al. 2011). This method is based upon the original Ellman et al. (1961) method, to determine cholinesterase activity using a portable analyzer among pesticide workers (McConnell et al. 1992).

Statistical analysis

SAS (version 9.3, SAS Institute, Cary, NC) was utilized for statistical analysis. The statistical significance level was set at .05. To construct a longitudinal model that could detect overall biomarkers and NB changes over time, a mixed effects model was used via PROC MIXED. Different within-subject covariance structures, such as compound symmetry (CS), unstructured, Toeplitz, and autoregressive, were employed for each NB measure. The likelihood ratio test, together with the Akaike information criterion (AIC) and Bayesian information criterion (BIC), were used for model selection (Fan and Li 2012). In all measures, CS was the covariate structure that yielded the smallest AIC. Linearity and outliers were checked by scatter and residual plots.

Candidate predictors for NB measures included urinary TCPy levels (a continuous variable measured at each time point); time as a categorical variable indicating the four time points; job status (categorical variable; applicator or nonapplicator); station of testing (Berket El-Sabea' or Al-Shohada); and age, BMI, and years of education at baseline. Due to the correlation between age and years of education, only years of education at baseline was kept in the model. In addition, a significant correlation was found between job status and TCPy levels. Thus, only the TCPy variable was used as a measure of exposure. TCPy levels were log-transformed since the AIC values are uniformly smaller than raw TCPy values (Morrell, Pearson, and Brant 1997). Hence, the regression model for NB measures included log TCPy, time, station, years of education, BMI, and log (TCPy) × time as predictors. For TCPy, AChE, and BChE, the predictors included time, job status, years of education, BMI, and time × job status. Main effects with *p* values >.1 were excluded from further model building. All tests were two tailed. Bonferroni correction of *p* values was applied to control for multiple comparisons.

Results

Demographic characteristics

Eighty-four adolescents (46 applicators and 38 nonapplicators) completed at least two of the four test sessions and were included in the study. There were no significant differences between the two groups regarding age, years of education, percent smokers, and distribution

among the two field stations, while applicators were significantly smaller in size than nonapplicators (Table 2). In agreement with these observations LaVerda et al. (2015) demonstrated that BMI was not significantly affected in pesticide applicators except for atrazine in the Agricultural Health Study (AHS). There were no marked changes in the demographic characteristics for participants at each of the four time points. Applicators worked an average of 3.3 years for the Ministry of Agriculture and reported applying pesticides 5 days a week for approximately 5 hr each day during the application season. Applicators of Al-Shohada reported working significantly more hours on the day of testing (3.8 ± 1.3) during the 2010 application season (Time 2) than Berket El-Sabea' applicators (1.6 ± 0.5). This was also seen in 2011, although the difference was not significant (Time 4), 3.3 ± 0.6 and 2.9 ± 0.9 , respectively.

Chorpyrifos metabolite in urine across the four time points

TCPy levels significantly changed from one time point to the next. A significant increase occurred at Time 2 following CPF application in 2010 compared to Time 1, a significant decrease to baseline levels at Time 3 prior to CPF application in 2011, and elevation again at Time 4 during pesticide application in 2011. The covariates in the model showed that applicators exhibited significantly higher levels of TCPy across the four time points than nonapplicators. A significant interaction between job status and time indicates that differences in TCPy levels between applicators and nonapplicators occurred during CPF application: Time 2 and Time 4. Years of education was also significantly associated with TCPy concentrations, and more years of education were correlated with lower levels of TCPy (Figure 1, Supplement Table 1).

Cholinesterase activity across the 4 time points

BChE activity also showed marked alterations across the four time points, demonstrating inhibition of cholinesterase activity following exposure. BChE activity decreased at Time 2 following application of CPF, rose at Time 3 after application ended, and then fell again at Time 4 during application of CPF (Figure 2a, and Supplement Table 2). Applicators displayed significantly lower levels of BChE activity than nonapplicators across the four time points. A further analysis of the interaction between time and job status indicated that marked differences between applicators and nonapplicators were only found during the application seasons, at Time 2 and Time 4. Unlike BChE, AChE activity did not show these changes where significant inhibition of AChE was observed only during CPF application in 2010 at Time 2 (Figure 2b; Supplement Table 2).

Correlation between CPF metabolite and cholinesterase

A significant negative correlation was found between TCPy and BChE ($r = -.67$, Figure 3a), controlling for time and job status. The degree of correlation between TCPy and AChE was less than that of BChE ($r = -.14$; Figure 3b). The fit plot in Figure 3 demonstrates the effect-response relationship between TCPy and both cholinesterase measures. Data indicate that BChE was more responsive to increasing levels of TCPy, and illustrate the inflection point at which a progressive inhibition of BChE started. The inflection point was $28.9 \mu\text{g TCPy/g creatinine}$ (which is 1.4 on the log scale in Figure 3a). The fit plot for AChE (Figure 3b) did not demonstrate this effect-response relationship.

NB performance across testing times and the impact of TCPy levels

NB performance improved over time, demonstrating learning or practice effects (Table 3, Supplement Table 3) which were reduced over time. Supplement Table 3 shows the least-square means of the four time points from the fixed effect model, and Table 3 presents the coefficients of change from each time point to the next (the middle column, Time effect) of each NB measure. Table 3 presents the improvement from Time 1 to Time 2 noted on 13 of the 16 measures, the number of measures continuing to show that improvement fell at Time 3 (7 out of 16) and also at Time 4 (3 out of 16). Only one NB outcome (continuous performance test-hit latency; CPT-hit latency) demonstrated a decrease in performance after exposure in 2010 (Time 2), which then improved at Time 3 prior to the application of CPF in 2011.

TCPy levels were significantly associated with seven NB outcomes (Table 3). Decrements in performance associated with greater TCPy levels were found in the following measures: continuous performance-corrected hit fraction (CPT-C-H-Fraction) and continuous performance-D-prime (CPT-D-Prime; attention function), time to complete trail making B (TrailB), symbol digit response latency (SDT-LAT; visual motor function), similarity (verbal abstraction function), average of right and left hand tapping (TAP-Aver; motor function), and Santana dominant hand (Santana-Dom; motor speed function) (Figure 4). Although not significant, other NB outcomes also showed a numerical decrement associated with elevated TCPy levels: visual motor integration (VMI) and digit span test forward (DST-F; attention function). Only one NB measure exhibited a significant interaction between time and TCPy levels: Santana dominant hand where the negative correlation between TCPy levels and Santana-Dom score occurred only at Time 4. The other covariates of NB performance included in the model were field stations and years of education. Participants from Al-Shohada performed significantly worse on CPT-C-H-Fraction, Santana-Dom and Santana non-dominant hands (Santana Non-Dom), TAP-Aver, VMI, and similarities measures than participants of Berket El-Sabea'. Years of education was also markedly, positively associated with digit span reverse (DST-R), TAP-Aver, and VMI measures.

Correlation between cholinesterase activity and NB performance

Partial correlation coefficients of both BChE and AChE with NB measures, controlling for time of testing, urinary TCPy levels, station, job status, and years of education, found a significant correlation for only two out of 16 NB measures: AChE was correlated with DST-F and Trail A, while BChE was correlated with CPT-Hit-Latency and Similarities (Supplement Table 4).

Discussion

Biological measures

Changes in OP metabolites across an application season among exposed subjects are previously reported by Crane et al. (2013). Crane et al. (2013) found that TCPy levels among both applicators and nonapplicators were elevated during CPF application, reached a maximal level at the end of the CPF application cycle, and then recovered to near baseline levels approximately 6 months after the end of the application season. The current study

expanded this time period into a second application season to examine the impact of repeated CPF application on TCPy levels. For both years, baseline levels of TCPy (Time 1 and Time 3) were similar and showed a rise following application of CPF (Time 2 and Time 4). Although other studies (Albers et al. 2004; Arcury et al. 2009; Garabrant et al. 2009) examined changes in exposure across the application season and noted increased exposure, this investigation is the first to measure exposure across two application seasons and demonstrate recovery to baseline levels after application ends. Cholinesterase activity, as expressed by both AChE and BChE levels, exhibited a similar pattern as TCPy levels. These findings also expand the outcomes of the 2010 season of the same study population (Crane et al. 2013). In the current study, BChE activity reflected alterations in exposure status and pesticide metabolite levels, where BChE activity was inhibited following the application of CPF for both seasons (Time 2 and Time 4) with a significant difference between degrees of blockade in applicators versus nonapplicators. BChE activity then recovered markedly to its baseline after the pesticide application season (Time 1 and Time 3). This confirms the observations from the 2010 season where both groups (applicators and nonapplicators) displayed significant inhibition during the CPF application cycle and started to recover after exposure stopped until it completely recovered after about 6 months (Crane et al. 2013). AChE did not exhibit this pattern, and the only significant change in AChE activity was inhibition following exposure in 2010 (Time 2). Similarly, Garabrant and colleagues (Garabrant et al. 2009) found that 58% of their exposed group experienced a 20% or greater decrease in BChE activity, relative to baseline, during the year of follow-up, while only 17% of controls had at least one measured BChE that was lower than baseline measure.

The higher sensitivity of BChE to increased urinary levels of TCPy relative to AChE was also readily apparent. The fit plots of both cholinesterase activity measures showed excessive blockade of BChE when TCPy reached an inflection point of 28.9 $\mu\text{g TCPy/g creatinine}$ (which is 1.4 on the log scale in Figure 3a). From Figure 3a, it appears that most study participants did not reach this limit, and inhibition of BChE occurred only in a small percentage of participants who displayed higher TCPy levels. This also replicates the findings of the 10-month study in 2010 of this population (Crane et al. 2013), the investigation of adult applicators in the same community (Farahat et al. 2011), and also the year-long examination of adult pesticide workers (Garabrant et al. 2009). The inflection points of Garabrant et al. (2009) (approximately 110 $\mu\text{g TCPy/g creatinine}$) and Farahat et al. (2011) study (average of 114 $\mu\text{g/g creatinine}$ during application) were higher than the inflection point in the current study (28.9 $\mu\text{g TCPy/g creatinine}$). This lower inflection point indicates enhanced vulnerability of adolescent applicators to high levels of CPF exposure.

NB outcomes

One difficulty with examining NB performance over multiple test sessions is that there may be learning or practice effects. While improvement in performance may be found in both exposed and control groups, often controls display greater improvement than exposed subjects (Baldi et al. 2011; Berent et al. 2014; Butler-Dawson et al. 2016; Nguyen et al. 2015). Despite the use of alternative forms, many NB measures in the current study showed initial improvement in performance, but the amount of improvement decreased over time (Table 3). In spite of this improvement in NB performance from one time point to the next,

greater TCPy levels in the current study were associated with decrements in approximately half of the NB measures assessing attention, visual motor, motor speed, and verbal abstract functions, indicating that improvement was not at the same rate for all participants, and performance of high exposed participants improved at a lower rate than low exposed individuals. The findings of our study are consistent with previous research examining NB outcomes in populations exposed to OPs (Mackenzie Ross et al. 2010; Rohlman et al. 2011). However, a strength of the current study is the extensive characterization of exposure. Previous studies may use various methods for classifying exposure.

Using TCPy level as a time-variant covariate in the longitudinal analysis model provided an accurate estimation of the impact of short-term exposure to CPF on NB outcomes over two seasons, rather than merely classifying the study group according to job status into applicators and nonapplicators or high- and low-exposed groups (Figure 4). The observed NB decrement which associated with high levels of CPF metabolite was noted in other research in the form of different rates of practice/learning effects for farmworkers and non farmworkers and their children in longitudinal follow-up studies (Baldi et al. 2011; Berent et al. 2014; Butler-Dawson et al. 2016; Nguyen et al. 2015). The significant relationship between TCPy levels and almost half of the NB measures affirms the findings of the 2010 study of the same study population. High-exposed participants demonstrated worse performance in 7 out of 22 NB measures than low-exposed participants (Rohlman et al. 2016). Further, when differences between high- and low-exposed groups were tracked across the study, it was noted that differences in NB outcomes between groups continued to rise during and after application. This cumulative effect of exposure among adolescents also demonstrates adverse NB effects associated with repeated exposure. The positive effect of education on NB performance was previously presented in other studies (Abdel Rasoul et al. 2008; Rohlman et al. 2007). Because all participants were enrolled in school, or had recently completed school, years of education was used as a surrogate of age. Similar to other investigations, data demonstrated that older subjects performed better on NB tests compared to younger ones. The negative association between years of education and TCPy levels (Supplement Table 1) may be due to additional education or experience that might protect them from exposure. Lower NB performance of Al-Shohada participants may be attributed to the more hours worked on the day of testing.

This relationship between TCPy and NB performance indicates the importance of quantifying the metabolite of CPF, TCPy levels in urine, as a measure of exposure. The prospective nature of our study and using TCPy as a quantitative measure of exposure provides more powerful evidence of the effect of chronic exposure to OP pesticides on NB performance compared to cross-sectional investigations that were not able to establish such a relationship (Maizlish et al. 1987; Rohlman et al. 2007; Steenland et al. 2000; Stephens et al. 1995; Stephens and Sreenivasan 2004).

The outcomes of the current study agree with other investigations that reported a nonsignificant relationship between cholinesterase activity and NB performance (Burns et al. 2013; Daniell et al. 1992; Farahat et al. 2003; Gomes et al. 1998). This raises the question regarding the applicability of using cholinesterase levels as a measure of effect of chronic exposure to OP pesticides which may include doses below those that inhibit cholinesterase

activity. Other mechanisms of OP chronic neurotoxicity were addressed in the literature and need to be considered for future work such as inhibition of the signaling cascades required in neuronal and hormonal inputs (Slotkin 2004) which leads to interruption of procedures of replication and differentiation of neurons, axonogenesis, and synaptogenesis (Curtin et al. 2006). Genetic difference in enzymatic activity and/or their expression is another suggested mechanism that may differentially affect the peripheral and central neurological outcomes (Hofmann et al. 2010). Oxidative stress was found to act as a contributor to chronic OP-induced neurotoxicity through inhibition of oxidative biomarkers including superoxide dismutase, glutathione reductase, glutathione-S-transferase, catalase, and glutathione peroxidase (Ali 2012). Another study found an association between exposures of flight crew members to OP pesticides and autoantibodies against glial and neuronal proteins that result from brain injury (Abou-Donia et al. 2013; Heutelbeck et al. 2016). Evidence indicates the need for more investigations to identify other mechanisms of deterioration in NB performance as a result of chronic exposure to OP pesticides (Burns et al. 2013; Li et al. 2012).

There are several limitations in our study, which include small sample size in which the highest participation was 66 adolescents at Time 1, late measurement of cholinesterase activity at Time 2, and confounding that may have occurred due to multiple exposures to OP pesticides and pyrethroids in the same season. In spite of the small size of the study sample, changes in biomarkers across application seasons and differences between applicators and nonapplicators were detected. Even though there is approximately a month and half between measuring cholinesterase activity and NB testing Time 2, which might result in some regeneration or recovery of cholinesterase activity, BChE still exhibited similar inhibition on consecutive years (testing Time 2 and Time 4) due to exposure to CPF. Regarding confounding by multiple exposures to CPF and also pyrethroids, applicators in each year worked in both cycles of CP application as well as the cycle of pyrethroid application of the relevant year. In addition, pyrethroid exposures are not clearly linked with NB deficits as is CPF (Horton et al. 2011; Oulhote and Bouchard 2013). Consequently, pyrethroids would require a relatively strong relationship with NB deficits to reasonably be considered as a confounder, even if it is correlated with CPF exposure. Despite these limitations, the study has certain strengths including (1) longitudinal follow-up of the study cohort, (2) using standardized methods to measure biomarkers and NB performance, (3) longitudinal analysis of data, and (4) availability of information regarding specific pesticides that were applied. This study was the first to examine adolescent pesticide applicators before and during exposure across two consecutive seasons, and enabled us to determine alterations in biomarkers of exposure (urine TCPy) and effect (cholinesterase activity), and NB performance across four times of testing.

Conclusions

In conclusion, adolescents working in pesticide application with the Egyptian Ministry of Health experienced an increase of TCPy excretion during exposure at both pesticide application seasons, and recovery was found after exposure ended in 2010. BChE as an effect biomarker was inhibited during exposure and recovered several months following exposure. Although the learning and practice effect on NB outcomes were observed,

estimating TCPy at four times points provided a quantitative measure of exposure that was a key factor in detecting a negative impact on NB performance. There is a need to continue examining the neurological performance of adolescent pesticide applicators across multiple years to determine the impact of exposure over time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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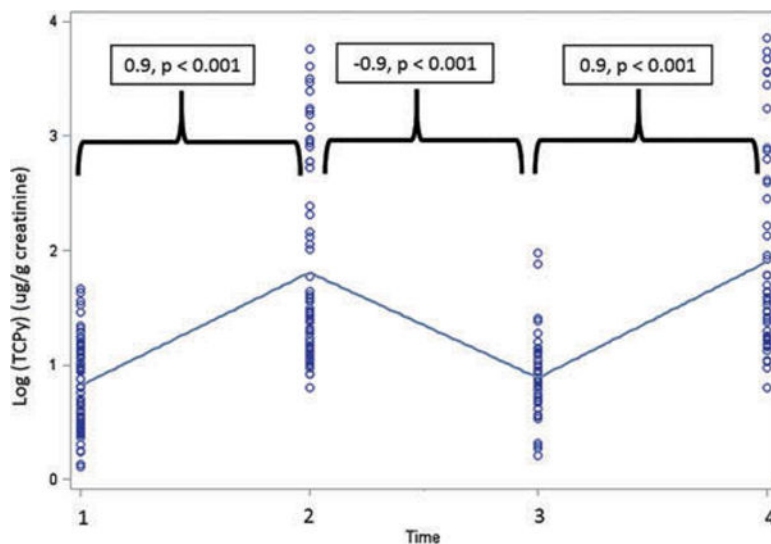


Figure 1.

Changes in urinary TCPy levels across the four time points of study ^{a, b}. ^a The model included time ($F= 58.1, p < .05$), job status (applicators vs. nonapplicators) ($F= 25.2, p < .05$), time \times job interaction ($F= 8.65, p < .05$), and years of education ($F= 9.4, p = .05$). ^b The numbers on the figure indicate the differences in least-square means between time points and their p values.

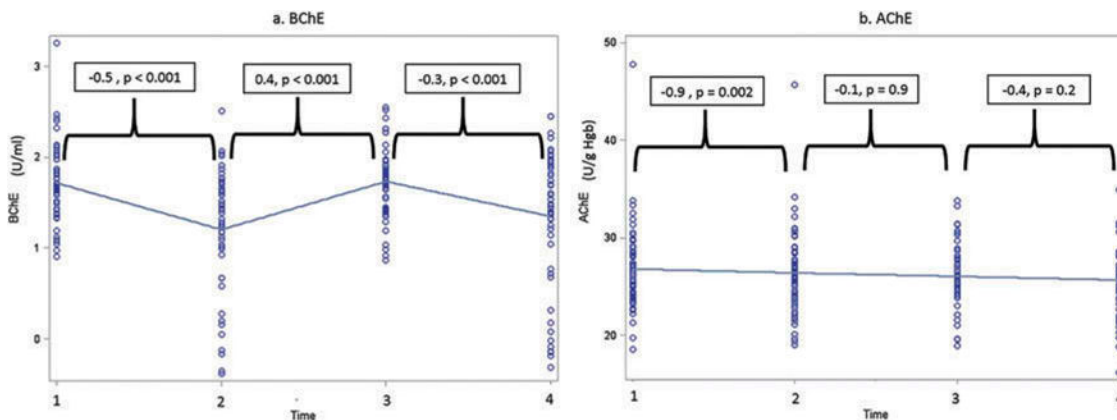


Figure 2. Changes in blood cholinesterase activity across the four time points of study^{a,b}. ^aThe model for BChE included time ($F= 17.0, p < .05$), job status (applicators vs. nonapplicators) ($F= 8.3, p= .05$), and time \times job interaction ($F= 6.4, p < .05$). The model for AChE included only time ($F= 17.0, p < .05$) as p values of other covariates were more than .1. The numbers on the figure indicate the differences in least-square means between time points and their p values.

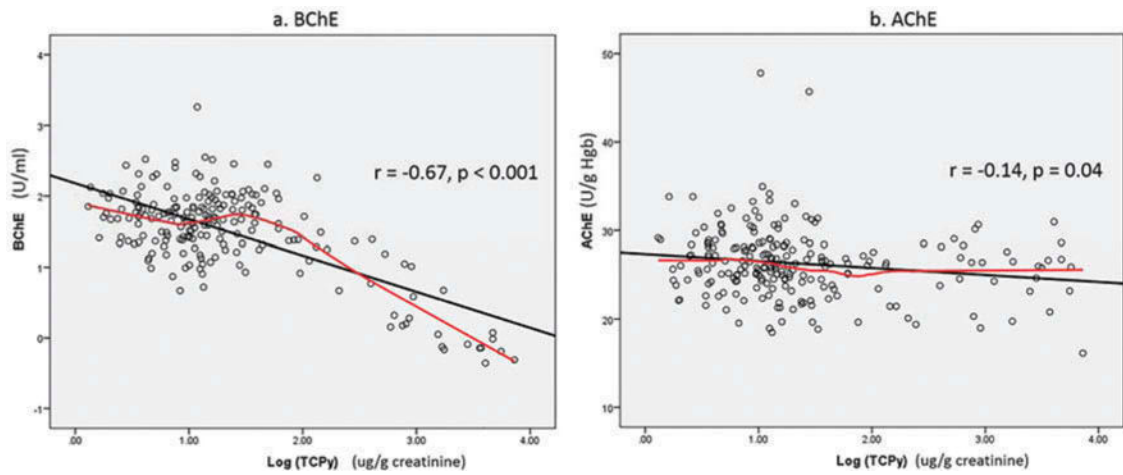


Figure 3.

Correlation and fit plots of blood BChE and AChE with urine TCPy^a. ^aThe straight lines in the graphs point to the correlation between cholinesterase and TCPy, while the bent lines point to the fit plots between cholinesterase and TCPy. The fit plots of BChE show progressive inhibition of BChE started when TCPy levels reach 28.9 μg TCPy/g creatinine (which is 1.4 on the log scale). The fit plots of AChE and TCPy do not show such a relationship.

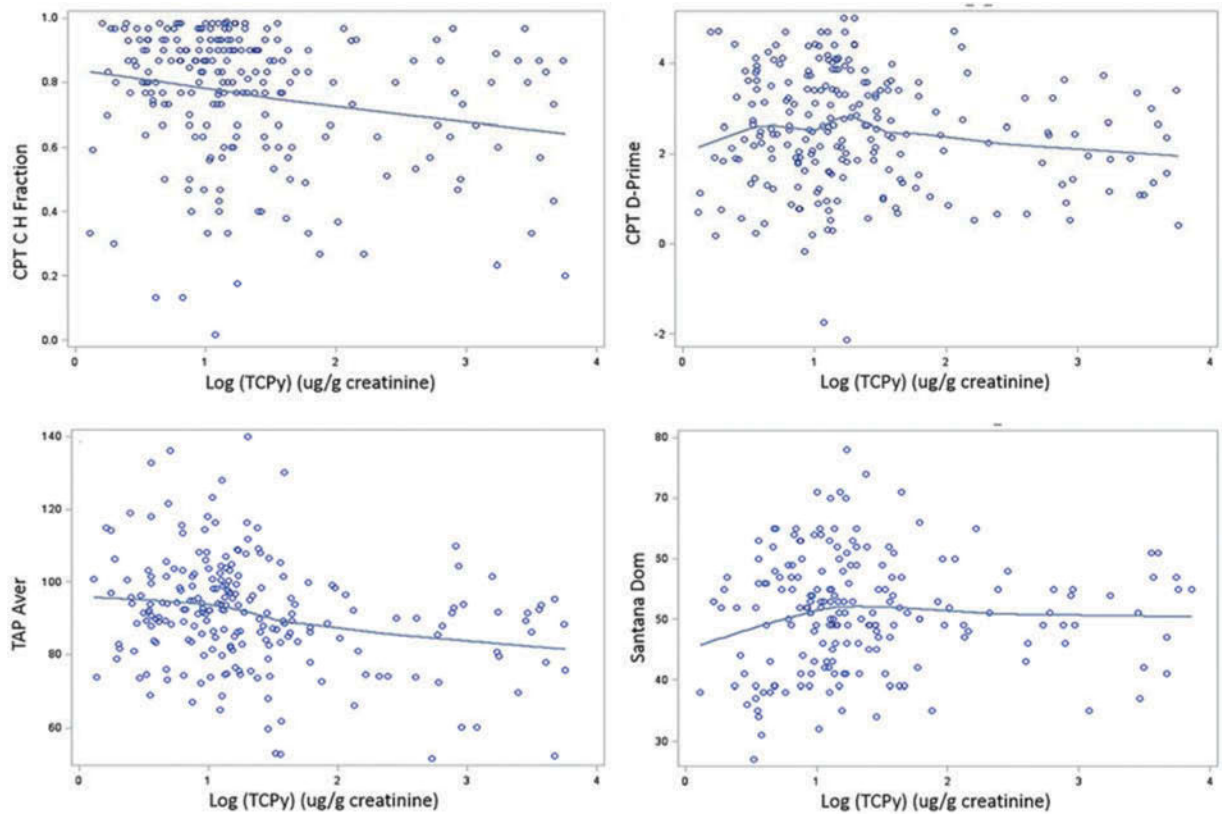


Figure 4. Fit plots of neurobehavioral measures and urine TCPy levels^a. CPT-C-H-Fraction, continuous performance test-corrected hit fraction; CPT-D-Prime, continuous performance test D-prime; Santana-Dom, Santana dominant hand; TAP-Aver; tapping average of right and left hands. ^aThe progress of the fit plots indicates the negative correlation between neurobehavioral measures and TCPy levels.

Table 1

Schedule of Application and Testing of the Four Time Points of the Study.

Time points	Date	Number of participants	Chlorpyrifos application periods
1	June 2/3, 2010	66	
2	July 14/15, 2010 ^a	65	June 23–July 17
3	May 28/30, 2011	50	
4	July 12/13, 2011	52	June 17–July 18

^aThere was no cholinesterase activity testing this time, the closest one was September 4 and 5, 2010.

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Table 2

Demographic Characteristics of the Study Participants.

	Nonapplicators (n = 38)	Applicators (n = 46)
Age (mean \pm SD)	16.7 \pm 2.3	16.2 \pm 1.7
Years of education (mean \pm SD)	10.0 \pm 1.7	9.9 \pm 1.9
BMI (mean \pm SD)	22.0 \pm 3.6	20.4 \pm 2.4*
Smokers, n (%)	4 (11.1%)	2 (4.3%)
Station [n (%)]	22 (57.9%)	26 (56.5%)
Berket El-Sabea'	16 (42.1%)	20 (43.5)
Al-Shouhada		

* p = .05.

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Table 3

Findings from the mixed-effect model examining changes in neurobehavioral outcome measures across the four time points, and the impact of TCPy levels, field stations, and years of education on neurobehavioral performance^{a,b}.

Function	Test	TCPy effect (β ± SE)	Time effect, differences of Least Square Means					Station (β ± SE) ^c	Years of education (β ± SE)
			Overall F-value	T1-T2 (β ± SE)	T2-T3 (β ± SE)	T3-T4 (β ± SE)	T1-T4 (β ± SE)		
Memory	BVRT		6.1**	1.0 ± 0.3*	-0.25 ± 0.3	0.1 ± 0.3			
Attention/short memory	DST-F	-0.3 ± 0.1#	2.8*	0.6 ± 0.2*	-0.3 ± 0.3	-0.1 ± 0.3			
	DST-R		0.1					0.1 ± 0.06*	
Sustained attention	CPT-Hit latency		2.8*	-25.4 ± 10.7*	27.5 ± 11.3*	-15.8 ± 11.7			
	CPT-C-H-Fraction	-0.06 ± 0.02*	1.6				0.1 ± 0.03*		
	CPT-D-Prime	-0.35 ± 0.2*	6.2**	0.7 ± 0.2**	-0.2 ± 0.2	0.3 ± 0.2	0.5 ± 0.2		
Motor speed/coordination	TAP-Aver	-2.7 ± 1.3*	8.2**	3.8 ± 2.1	4.3 ± 2.4#	0.3 ± 0.3	8.2 ± 2.4*	1.9 ± 0.7*	
	TAP-Alter		1.1						
	Santana-Dom	-2.4 ± 1.0*	26.4**	9.2 ± 1.6**	1.6 ± 1.7	3.9 ± 1.7*		4.9 ± 1.2*	
	Santana Non-Dom		92.3**	7.0 ± 1.0**	7.4 ± 1.0**	0.7 ± 1.0		2.7 ± 1.1*	
Visual motor	SDT-LAT	-211.7 ± 68.9**	25.9**	529.1 ± 125.3**	141.3 ± 130.2	81.6 ± 111.6			
	Trail A		78.5**	16.2 ± 1.7**	4.3 ± 1.7*	3.7 ± 1.6*			
	Trail B	-6.8 ± 2.3*	47.4**	30.7 ± 4.5**	8.8 ± 4.4*	7.2 ± 4.5			
	VMI	-0.5 ± 0.3##	38.5**	2.5 ± 0.5**	1.3 ± 0.5*	0.05 ± 0.5	1.2 ± 0.4*	0.4 ± 0.1*	
Perception	Block Design		109.8**	8.2 ± 0.7**	1.9 ± 0.7*	1.6 ± 0.7*			
Verbal abstraction	Similarities	-1.0 ± 0.4*	30.5**	5.0 ± 0.7	-0.1 ± 0.7	0.8 ± 0.7	1.4 ± 0.7*		

BVRT; Benton Visual Retention Test, CPT-C-H-Fraction; Continuous Performance Test- Corrected Hit Fraction, CPT-D-Prime, Continuous Performance Test D-prime, CPT-Hit-Latency; Continuous Performance Test - Hit Latency, Digit Span Reverse Test, DST-F; Digit Span Forward Test, DST-R; Santana-Dom; Santana Dominant Hand, Santana non-Dom; Santana Non-Dominant Hand, SDT-LAT; Symbol Digit Latency, Trail A; TAP-Alter; Tapping alternating hands, TAP-Aver; Tapping Average of Right and Left Hand, Trail A; Trail Making A, Trail B; Trail Making B, VMI; Visual Motor Integration.

^aThe only significant interaction of time and TCPy levels was in Santana dominant (F = 4.1, p = 0.009).

^bThe sign for estimates is standardized, where (+ve) sign indicates improvement in performance and (-ve) indicates decrement in performance.

^cFor the reported measures, Al-Shohada participants are significantly lower than those of Berket El-Sabeat.

p = 0.07.

p = 0.06;

p < 0.0001;

p < 0.05;
*

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